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Note

The action of *Bacillus subtilis* liquefying amylase on 6-deoxy-6-iodoamylose*

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A series of 6-deoxy-6-iodo-amyloses of various degrees of substitution (d s) have been prepared as substrates for a study of the action of amylases on modified substrates The samples having high d s values were so insoluble that they were not useful for the enzyme studies Preparations having d s 0 037–0 07 were used in determining the structure of the smallest iodinated fraction isolated The results reported here were obtained primarily with material of d s 0 037 (see Experimental)

RESULTS

A sample of 6-deoxy-6-iodo-amylose was treated with crystalline *Bacillus subtilis* amylase under conditions that would yield a limit hydrolyzate Paper chromatography of the hydrolyzate failed to reveal 6-deoxy-6-iodo-D-glucopyranose, even when relatively large aliquots of the digests were used Two components having R_G 0 51 and R_G 0 79 (solvent *A*) were observed to migrate between D-glucose (R_G 1 0) and maltose (R_G 0 33) Maltotriose, maltotetraose, and an oligosaccharide overlapping and trailing the maltotetraose spot were also detectable This slowest-moving oligosaccharide gave a zone extending almost to the origin, and it gave a darker spot when samples of higher d s values were used as substrates In a separate experiment, the sections containing the fractions suspected of being maltose, maltotriose, and maltotetraose were removed, extracted, subjected to the action of the glucamylase, and chromatographed with solvent *A* D-Glucose was the only product, showing that no iodinated fragments had been concealed in these sections The section migrating slower than maltotetraose, when treated with the same enzyme, yielded glucose plus some slow-moving, iodine-containing oligosaccharides

Multiple preparative paper-chromatography, with Whatman No 17 and 3MM papers and solvent *A* yielded the fraction having R_G 0 79, and a very small amount of the minor component having R_G 0 51 Each of these was subjected to the action of the same glucamylase, which had previously been shown to catalyze the hydrolysis of 6-deoxy-6-iodo-maltose (R_G 0 98) to monomers, and to have no activity on 6′-deoxy-

6′-iodo-maltose The enzyme mixture had no activity on the minor component, but did hydrolyze the R_G 0 79 fraction to glucose and 6′-deoxy-6′-iodo-maltose (R_G 1 6)

These results suggested that the major component might be a trisaccharide containing two D-glucose residues and an iodinated D-glucose residue The three possible structures are 6^1-deoxy-6^1-iodo-maltotriose (**1**), 6^2-deoxy-6^2-iodo-maltriose (**2**), or 6^3-deoxy-6^3-iodo-maltotriose (**3**)

The action of the glucamylase had suggested that the unknown was not **1**, because that trisaccharide would probably have been hydrolyzed to D-glucose and 6-deoxy-6-iodo-D-glucopyranose

The component having R_G 0 79 gave a crystalline peracetate whose mass spectrum showed that this major component was indeed a trisaccharide of two D-glucose residues and one iodinated D-glucose residue, as revealed by the presence of an ion *m/e* 991 ($M^+ - CH_3CO$) and an ion *m/e* 975 ($M^+ - CH_3COO\cdot$), where M = 1034 mass units The presence of a peak *m/e* 687 showed that the peracetate was either **2** or **3** because removal of the reducing-end glycosyl residue from **1** would have yielded an ion having *m/e* 619

Cleavage of the remaining glycosidic bond would yield an ion of *m/e* 399 had the original structure been **3** or *m/e* 331 had it been **2** Although there was a small peak having *m/e* 399, its intensity was only 0 22%, as compared with the large peak at *m/e* 331, which was taken as relative intensity = 100% The mass spectrum of the peracetate of the major component thus indicated it to be 6^2-deoxy-6^2-iodo-maltotriose

DISCUSSION

The absence of an iodinated monomer in the enzyme hydrolyzate is consistent with results obtained with this enzyme on 6-*O*-methylamylose[1], 2(3)-*O*-methylamylose[2], and 6-amino-6-deoxyamylose[3] The modified glucose is always found as part of an oligosaccharide Action of crude fungal glucamylase on 6-*O*-methylamylose of low d s gives 6′-*O*-methylmaltose The same mold enzyme mixture produces a similar product, 6′-deoxy-6′-iodo-maltose, from the iodinated substrate used in this work

The experiments on 6-deoxy-6-iodo-amylose show that this crystalline bacterial amylase produces a trisaccharide, chromatography has indicated that a trisaccharide is the smallest ninhydrin-positive fraction produced by the same enzyme on 6-amino-6-deoxy-amylose[3]

The fact that the trisaccharide liberated in the present experiments has the iodinated D-glucose residue in the middle suggests that the iodine atom has the same effect as the branch point in amylopectin, where 6^2-(α-maltosyl)maltotriose is the smallest branched molecule produced[4] It seems that this bacterial amylase cannot catalyze cleavage on either side of the modified D-glucose residue

EXPERIMENTAL

Materials — Corn amylose (A E Staley Co), crystalline *Bacillus subtilis* amylase (Enzyme Development Corp.), and the mold enzyme mixture termed glucamylase (Clinton Corn Processing Co) were commercial samples The preparations of 6-deoxy-6-iodo-maltose and 6′-deoxy-6′-iodo-maltose are described in another communication[5]

Methods — Descending paper chromatograms were developed with 4.1 1 (v/v) butyl alcohol–ethanol–water (solvent *A*), and 6 4 3 (v/v) butyl alcohol–pyridine–water (solvent *B*), with visualization by the alkaline silver nitrate method of Trevelyan *et al* [6] Thin-layer chromatograms were conducted on prepared Silica Gel G plates (Analtech) by using 9 7 4 (v/v) ethanol–toluene–diethyl ether with spot development effected by spraying with 5% H_2SO_4 in ethanol, and heating at 105° Mass spectra were recorded with Varian Associates Atlas 311 instrument equipped with a direct-insertion probe The source temperature was 180°, and the ionizing potential was 70 eV

Modified substrates — 2,3-Di-*O*-acetylamylose and 6-*O*-tosyl-2,3-di-*O*-acetylamylose were prepared according to the procedures of Whistler and Hirase[7], but by using shorter reaction times for the 6-*O*-tosyl product to obtain lower d s values

2,3-Di-*O*-acetyl-6-deoxy-6-iodo-amylose was prepared by adding sodium iodide (35 g) to the tosylated products (12 5 g) in 800 ml of *N*,*N*-dimethylformamide The solution was heated for 10 h at 95°, and poured into 2 liters of 50% aqueous methanol in a blender The suspension was filtered, and the residue suspended successively in water, methanol and diethyl ether, with suction filtration at each step The product was dried under vacuum for 5 h at 40°, yield 9 5 g

6-Deoxy-6-iodo-amylose was prepared from the acetylated product by stirring 4 5 g of it in an excess of 0 15M sodium methoxide in anhydrous methanol The mixture was stirred for 16 h, filtered, and suspended successively in anhydrous methanol (two times) and in diethyl ether (two times), with suction filtration after each washing It was dried at 40° under vacuum, yield 2 95 g (Found I, 3 10%, corresponding to d s = 0 040)

The 6-deoxy-6-iodo-amylose (50 mg) was refluxed in 1 5 ml of 0 75M sulfuric acid for 10 h, and the solution was neutralized with Amberlite IR-400 (carbonate form), concentrated, and an aliquot evaporated to dryness Trimethylsilyl ethers were prepared by adding hexamethyldisilazane and chlorotrimethylsilane to the dried sample in *N*,*N*-dimethylformamide G l c analyses were conducted in duplicate on an HP-5750 chromatograph equipped with a hydrogen-flame detector, and a 6 ft × 1/8 in column of 20% SE-30 on Chromosorb W The flow rate of helium gas was 28 ml/min, and the temperature was programmed from 125 to 270°, at 10° per min Comparison of the areas under the peaks with standards indicated d s = 0 034

Enzyme hydrolyses — These were conducted on a 1 0 g sample of 6-deoxy-6-iodo-amylose that had been wetted with butyl alcohol and dissolved in boiling water The pH was adjusted to pH 5 8 with a minimum amount of acetate buffer, and 20 mg of crystalline *Bacillus subtilis* amylase was added A test with iodine gave no color

after less than 30 sec After 72 h at room temperature the solution was concentrated to 25 ml and centrifuged to remove a small amount of precipitate (5 mg) The solution was further concentrated by lyophilization, and chromatographed on Whatman No. 17 paper with solvent *A* for 22 h The strip migrating between glucose and maltose was eluted, concentrated, and rechromatographed on Whatman No 3 MM paper for 144 h with solvent *A* The two components having R_G 0 51 and R_G 0 79 were isolated and found to be chromatographically pure with both solvent systems *A* and *B*

Small amounts of these two components were subjected to the action of a dialyzed sample of the mold enzyme mixture, and then chromatographed on Whatman No 3 MM paper with solvent *A* to afford the results previously discussed, these results were confirmed by t l c

The experiment to prove that the maltose, maltotriose, and maltotetraose fractions were free of iodinated saccharides was conducted on a small sample (50 mg), of the substrate, with chromatography and isolation from Whatman No 3 MM paper

The fraction having R_G 0 79 was converted into its peracetate with acetic anhydride–pyridine in the conventional manner

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